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Alison Brett Gruen

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Iron Uptake in Human Keratinocytes Exposed to Photodegraded Nifedipine. *Alison B. Gruen, Jing Zhou, Kathryn Morton*, and Leonard M. Milstone, Depts. Dermatology and Radiology*, Yale University School of Medicine, and VA Medical Center, West Haven, CT. and Portland, OR*.*

Photodegraded nifedipine (PDN) has been shown to increase uptake of non-transferrin bound iron in erythroid cells. We investigated the ability of photodegraded nifedipine to mediate iron transport in human keratinocytes with the underlying goal of exploiting the process of epidermal desquamation for the purpose of eliminating toxic chemicals such as iron. Nifedipine was exposed to direct outdoor daylight for 1 hour to allow for the formation of the active nitroso metabolite. The adequacy of photo-degradation was confirmed by thin-layer chromatography on silica gel. Keratinocytes were incubated at 37°C with 15 uM ^{59}Fe and either 25 uM PDN or ethanol. The monolayer was trypsinized and centrifuged through a dibutylphthalate:paraffin oil column. Cell pellets were collected for gamma counting and protein assay. PDN stimulated a 20-fold increase in iron uptake in 30 minutes relative to controls. The keratinoctye's ability to accumulate and retain iron was tested by pulsing cells for 1 hour in 25 uM PDN and 20 uM Fe on four successive days. After a four day washout, the total iron content of keratinocytes - measured by inductively coupled plasma emission spectroscopy - was 3-fold greater in PDN-treated dishes than in controls. The effect of time, dose, and cellular differentiation were similarly investigated. PDN provides an effective method for loading iron into keratinocytes.

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Table of Contents

Introduction

A primary function of the epidermis is to provide a renewable barrier, particularly to water loss. The epidermal barrier is maintained in delicate equilibrium between the production and loss of its composite cells. As spent keratinocytes and barrier remnants desquamate from the surface, regeneration of cells at the basal layer ensures a continually renewing supply. The epidermis is morphologically and functionally stratified. Proliferating cells are restricted to the basal or innermost cell layer. As these cells progressively differentiate and traverse the epidermis, they are shed or desquamated from the most superficial layer of the epidermis, the stratum corneum. The transition from basal cell to squame normally occurs in 28 days. In hyperproliferative disorders of the epidermis, like psoriasis, epidermal renewal is accelerated 6-fold.

Epidermal renewal naturally has metabolic consequences in the form of the cellular contents lost to the environment with desquamated cells. One gram of dry weight is desquamated daily from normal skin, and up to 12-15 grams per day in psoriatic erythroderma (1). Studies on protein and mineral content of scale suggest that erythrodermic losses are insufficient to cause negative nitrogen balance (1), but may result in iron deficiency by cellular iron losses (2). The degree to which the epidermis participates in body iron balance is under-appreciated. 20-25% of absorbed iron is eliminated daily through the skin (3, 4, 5). These epidermal losses result mainly from cellular desquamation rather than sweating (6, 7).

Much of our present understanding of epidermal mineral loss comes from studies performed in the 1950s, 60s, and 70s. There is abundant information on the potential burden of minerals but few studies on the potential metabolic benefits. Certainly, the boundaries of epidermal function have been challenged. Through genetic engineering the epidermis has been modified for the secretion of hormones and growth factors (8), modulation of immune function (9) and the inactivation of toxic metabolic products. (10). In the presence of such innovation, it is surprising that one of the most basic qualities of the epidermis—desquamation—was overlooked. The unique process of desquamation makes it an appealing site for further investigation. Essentially, desquamation represents a fixed route of loss to the environment. Certainly there are medical diseases and conditions that would benefit from such an alternative excretory pathway. The question then arises: Could desquamation be exploited to eliminate toxic chemicals like lead, iron, or copper from the body?

For several reasons, iron and diseases of iron overload represent a sensible preliminary model to investigate this possibility. Iron overload presents a clinically significant problem with imperfect therapies; there is an abundance of knowledge about body iron homeostasis; and, such research would advance our understanding of the particular role of the epidermis in iron metabolism. Because this represents a novel approach to these diseases and to desquamation in general, there is no specific scientific literature. A wealth of information, however, does exist on body iron metabolism and epidermal desquamation.

Iron Metabolism – Then and Now

Iron actually has a long and glamorous history. It's rise to historical preeminence came with the advent of the Iron Age. Unbeknownst to many, this evolution precipitated the fall of the ancient Egyptian civilization. Much of Egypt's power was afforded by their control of large deposits of copper (the main ingredient in bronze) in the Sinai Peninsula. By the thirteenth century B.C. other societies had discovered and successfully employed iron, a harder and more abundant element, in tools and weapons allowing larger, better equipped armies. The copper-clad Egyptian army was unable to meet the challenge of its new foe (11).

This heroic motif in iron chemistry reappears centuries later in mythology. In the Greco-Roman tradition, iron was associated with the god of war, known to the Greeks as Ares and to the Romans as Mars. Other myths, including that of Jason and the Argonauts, are rife with images of iron as a source of strength, potency, and healing (11).

On a softer side iron, or rather its deficiency, created the romantic, though sickly image of "lovesickness" because of the demographics of its sufferers. Naturally, marriage was thought to be the best salve. The ultimate causal association between iron deficiency and the anemic appearance of centuries of young, malnourished women, unfolded slowly and with amusingly clever misdiagnoses. Because of the unexplained green tint that iron deficiency gave pale skin, it was medically described as 'chlorosis' (from the Greek word for *green*) or more popularly, "greensickness." Writers from Hippocrates to Shakespeare alluded to the disease. It was not until the late 17th Century that the link between iron deficiency and 'chlorosis' was elucidated. Thomas Sydenham of London in late 17th century is credited with the discovery (11).

One of the predominant themes in iron metabolism is of duality, of iron as both an essential nutrient and a toxin. Early insight into the contrasting properties of iron are present in the first encyclopedia The Historie of the World, 1st century A.D. by Gaius Plinus Secundus. An old English translation dating to 1601 notes:

> "It remaineth now to discourse of ... yron, a mettal, which wee may well say is both the best and the worst... in the world: For with the help of yron we breake up and ear the ground, we plant and plot our groves, we set our hortyards... by means of yron and steele we build our houses, new quarries...yea and in one word, we use it to all other necessarie uses of this life. Contrariwise, the same yron serveth for warres, murders, robberies...this I take to be the wickedest invention that ever was devised by the head of man..." (12)

The paradox of iron metabolism is apparent still. It is an essential nutrient required by all organisms for growth and metabolic pathways. Iron dependent processes include DNA synthesis, ATP generation, and hemoglobin and myoglobin function. However, iron is also a potential toxin, capable of catalyzing free radical formation resulting in damage to DNA and cellular membranes. The unique chemistry of iron presents a challenge as well. Two valencies account for iron's divergent properties: the participation in biologic redox reactions vs. a tendency towards free radical formation, the solubility of some of its compounds vs. the insolubility of others. In an oxidizing environment, iron exists in the largely insoluble ferric form, making its transport, solubilization and storage a matter of importance for all organisms (13, 14).

To insure appropriate body iron content, body iron metabolism is well balanced. Under normal circumstances, iron does not accumulate in the body. Of the average ingested iron load of 10-20 mg/day, only 1 mg is absorbed, an amount which increases

with vitamin C or alcohol. To avoid accumulation of iron, 1 mg per day must therefore be excreted. Approximately 0.6 mg is lost in feces, 0.2 – 0.3 mg is lost from the skin, and less than 0.1 mg is lost in urine (15). During the years between menarche and menopause, women's iron losses increase to 2 mg/day with a compensatory increase in absorption to about 1.5 mg/day (16,14). Total body iron content is large relative to seemingly small daily exchanges. Normal body iron stores are approximately 4 g in men, 3 g in women. These stores are balanced among functional compounds, storage complexes and transport chelates. The distribution of iron in tissue, as adapted from a recent NEJM review on iron metabolism, is shown in Figure 1 (17). Most of this iron, between 35-40 mg per kg body weight, represents functional iron, 75% of which is in hemoglobin. Another 0-20 mg per kg iron exists as a reserve form in ferritin and hemosiderin—two major iron storage proteins (15).

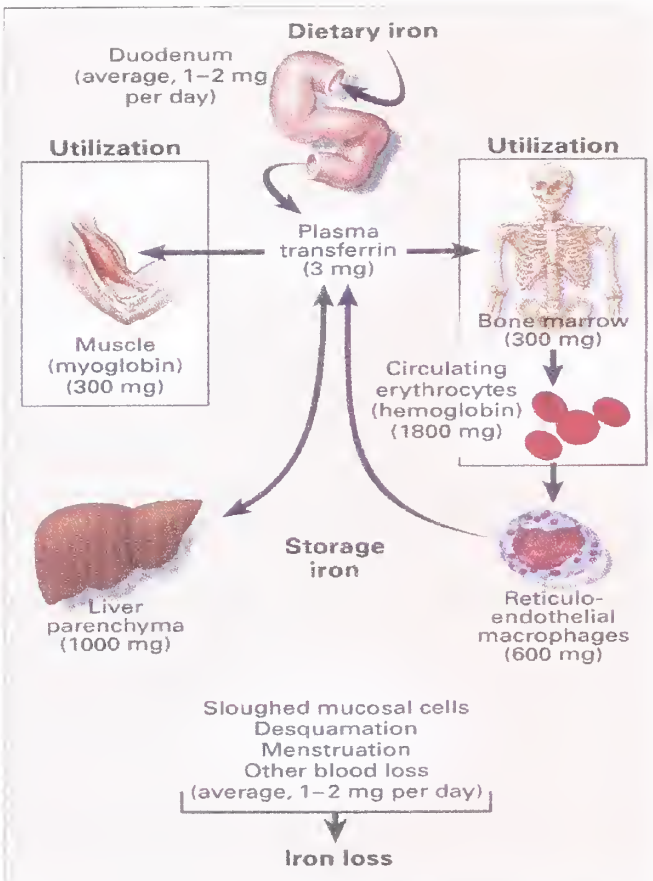

Fig. 1. Distribution of iron in adults. In the balanced state, 1 to 2 mg or iron enters and leaves the body daily. Dietary iron is absorbed in the duodenum and then circulates in the plasma bound to transferrin. Most of the iron in the body is incorporated into hemoglobin in erythroid precursors and mature red cells. Approximately 10-15 percent is present in muscle fibers (in myoglobin) and other tissues (enzymes and cytochromes). Iron is stored in parenchymal cells of the liver and reticuloendothelial macrophages. These macrophages provide most of the usable iron by degrading hemoglobin in senescent erythrocytes and reloading ferric iron onto transferrin for delivery to cells.

Diseases of Iron Overload

In certain disease states this homeostasis is challenged and iron accumulates abnormally in the body. Systemic iron overload results in the deposition of toxic

amounts of iron in parenchymal cells. The intracellular iron pool expands uncontrollably surpassing not only transferrin binding but also the sequestering capacity of ferritin. The emergence of this unbound toxic form of iron promotes the formation of free hydroxyl radicals that damage cellular membranes, DNA and proteins (18). Diseases resulting from iron overload can be either primary or secondary—resulting from a recessive disease of iron transport or from excess dietary iron and diseases requiring frequent blood transfusions.

Hereditary hemochromatosis (HH), unbeknownst to many, is the most common genetic disorder in the U.S. Caucasian population. The gene frequency in this population is 5-10%. A homozygote genotype occurs with a frequency of one in 250 persons, though there is variable phenotypic expression (18). Ninety percent of Caucasian hereditary hemochromatosis patients are homozygous for the same mutation (C282Y) in the HFE gene—the mutant protein in HH. Studies in HFE knockout mice and in B2-microglobulin knockout mice (a mutation which inhibits cell surface expression of HFE) document a pattern of iron overload similar to that in hemochromatosis (19, 20, 21, 22). The function of the HFE protein in iron absorption is unclear. One recent study showed that the wild type HFE protein decreases the affinity of the transferrin receptor for transferrin, raising the possibility that a loss of down-regulation of transferrin-mediated iron transport may be responsible for HH (23). Other studies reach different conclusions as to the nature of HFE participation (24). The exact interaction between HFE, the transferrin receptor, and other cellular iron transporters awaits further illumination.

The end result is a failure to appropriately regulate intestinal iron absorption. Patients with hemochromatosis absorb 2 to 3 times the amount of daily dietary iron as

normal individuals, approximately 4 mg per day. Because there is no known excretory mechanism for excess iron, its levels in the body—normally constant—begin to rise. Parenchymal iron deposition occurs as transferrin saturates. An elevation of transferrin saturation is usually present by adolescence, but is rarely detected before clinical symptoms occurring decades later prompt an investigation. The clinical picture in HH is one of slow, insidious progression with clinical manifestations occurring after total body iron approaches 20 grams—the normal level being 3-5 grams (15). In affected men this occurs by ages 40-60. Women present later because of a physiologic iron losses during menstruation, childbirth, and lactation (25). Initial manifestations include fatigue, skin hypergimentation, arthralgias, and erectile dysfunction. With disease progression, elevated hepatic iron causes tender hepatomegaly, then ultimately cirrhosis and, not uncommonly, hepatocellular carcinoma—an outcome responsible for 10-30% of hemoochromatosis-related deaths (26). Iron deposition in endocrine organs results in hypogonadism, hypothyroidism, and diabetes. In the heart it causes cardiomyopathy (27).

The progressive nature of the disease makes early recognition and initiation of therapy imperative. Unfortunately the classic clinical description of the disease as 'bronze-diabetes' is misleading because it depicts the end-stage of disease where organ failure is imminent (27). As a result, physicians often miss an earlier, and perhaps, life-saving diagnosis. A landmark study by Niederau, 1985 determined that the survival of HH patients was the same as age- and sex-matched controls without the disease if treatment was initiated before the development of cirrhosis or diabetes (26). The treatment for hemochromatosis, therapeutic phlebotomy, has been used medically for

centuries and for hemochromatosis since the 1950s. Initially 500 mL of blood are removed per week until ferritin is less than 10-20 ug/L. Then patients undergo 4-8 phlebotomies per year for life to maintain ferritin levels less than 50 ug/L (27). The therapy is safe, economical, and effective.

Secondary hemochromatosis is most often seen in the context of those diseases requiring therapeutic blood transfusions, like thalassemia and the hemoglobinopathies. Patients with these diseases accumulate excess iron in two ways. First, ineffective erythropoesis stimulates increased intestinal iron absorption. The second and more significant burden is the weekly transfusions that cause a condition known as transfusion siderosis. A single unit of blood contains between 200-250 mg of iron—a staggering amount compared to the normal 1 mg per day that is excreted. This iron, being derived from senescent erythrocytes, is first cleared by reticuloendothelial macrophages. Much of the iron is ultimately recycled to transferrin and delivered to parenchymal cells, producing the same pattern of overload as hereditary hemochromatosis. Myocardial siderosis is the most important cause of mortality in sub-optimally treated transfusional overload, possibly because of the rapidity of iron loading (28).

Therapeutic phlebotomy is precluded by the nature of the primary disease. Iron chelation therapy with desferroxamine has been used effectively since the early 1960s. Treatment involves continuous subcutaneous or intravenous delivery by portable pumps. Such a schedule is necessary to limit hepatic iron load to less than 15 mg per gram of liver, dry weight (29). Although desferroxamine is relatively non-toxic, excessive doses can cause skeletal growth abnormalities and sensorineural hearing loss (30). The major shortcomings of DFO therapy, however, are the high cost and the inconvenient and

uncomfortable mode of delivery. Oral chelators such as deferiprone have been developed, but are not universally effective and have complications (17).

There is clearly room for therapeutic intervention in the management of iron overload diseases. A convenient, non-toxic, inexpensive therapy would be a welcome replacement for desferroxamine in cases of transfusional overload. And although phlebotomy is safe and expedient for patients with hemochromatosis, there are hints that a steady, rather than intermittent therapy might be more appropriate. Clinical data and cellular iron metabolism support the notion of continuous therapy. While long term DFO treatment removes non-transferrin iron from the blood, on cessation of therapy it reappears rapidly, necessitating continuous therapy (31). There is also a physiologic advantage to removing cellular iron before it becomes durably bound up in ferritin or hemosiderin particles. Ferritin most efficiently releases iron when it is only partially bound with iron (14). Studies with Mossbauer spectroscopy support this feature by demonstrating that most iron mobilized by DFO is derived from small (< 0.3 nm), recently acquired iron particles. Larger particles, such as the iron core of ferritin molecules are conversely unavailable for DFO chelation (32).

Cellular Iron Metabolism

An understanding of the regulation of cellular iron metabolism helps elucidate the mechanisms of systemic iron overload. This knowledge is also critical in any therapeutic attempt to manipulate or else circumvent the pathologic patterns of parenchymal iron

accumulation. The homeostasis achieved in iron metabolism involves the participation of different transporters and storage molecules. Iron is deposited in two forms in tissue—ferritin and hemosiderin. Ferritin is the major storage protein for iron in the body; normally twice as abundant as hemosiderin. It can both accept iron in states of excess and release it in times of erythropoetic demand. This flux occurs most efficiently with iron in its reduced, ferrous state (33). Once in ferritin's protein core, however, the iron is oxidized and maintained in the ferric form. A single molecule of ferritin can store up anywhere from 2500 to 4500 atoms of ferric iron in this manner (34, 35). When ferritin becomes saturated with iron, its protein shell is partially degraded by lysosomal proteases to yield hemosiderin. Hemosiderin is an insoluble, ill-defined aggregate of iron oxide with organic constituents. It is up to 40% iron by mass. Compared to ferritin, it is a more durable sink for iron (14). This explains the prevalence of hemosiderin deposits in states of iron overload. As total tissue iron levels increase, so does the proportion of hemosiderin to ferritin. In liver biopsies of iron-overloaded patients, hemosiderin levels were 100-fold and ferritin only 10-fold above normal (36).

There are two major forms of iron transport in the body—one mediated by transferrin and one that is transferrin-independent. The function and regulation of the transferrin-mediated pathway is well characterized. Mammals evolved transferrin as a necessary solution to the evolutionary problem presented by ferric iron—a form which lacks both solubility and bioavailability. By transporting iron as a soluble diferric chelate between body compartments, transferrin presents iron in a form amenable to cellular uptake and responds to cellular iron needs. Uptake of transferrin bound iron occurs via receptor-mediated endocytosis. Upon binding to its high affinity cell surface receptor,

present on nearly all cell types, transferrin dimerizes. The transferrin-receptor complex is internalized. Following acidification of the endosome, the transferrin receptor (TfR) is recycled to the cell surface and iron is released into three cytosolic pools. It is utilized in heme proteins and metabolic enzymes, sequestered in ferritin, or enters a transient regulatory pool that controls iron levels in the cell (37, 38).

The regulatory form of iron maintains cellular iron balance by controlling the translation of ferritin, transferrin receptor (37, 13), and divalent metal cation transporter DMT1 mRNAs (39). The system is an example of post-transcriptional control, which allows a rapid and sensitive response to cellular needs for iron. When iron enters a cell it is bound by cytosolic iron binding proteins. This iron-protein complex affects translation by interacting with regions on the mRNA of both the TfR and ferritin called iron responsive elements (IREs). When iron binds the protein, the protein is released from the IRE. Release of the binding protein from the 3' IRE in the TfR mRNA destabilizes mRNA stability decreasing translation of the message into TfR protein and thus reducing transferrin-mediated iron uptake. Alternatively, release of the binding protein from the IRE in the 5' end of ferritin mRNA allows its translation, increasing synthesis of ferritin and intracellular iron storage (37, 40).

The type of regulation represented by the transferrin receptor ensures cellular iron homeostasis. Ward et al. was among the first to demonstrate that TfR number was regulated by intracellular iron content rather than ligand binding—a mechanism akin to LDL receptor regulation. Preincubation of fibroblasts with ferric ammonium citrate reduced the number TfRs per cell, whereas saturating levels of $Tf(Fe)_2$ or apo-transferrin had minimal effect on TfR expression (41).

Another mechanism of iron uptake, a non-transferrin-bound iron uptake system, was first recognized by Wheby and Crosby in 1963 and continues to be characterized (42). Its function and regulation varies widely from that of transferrin. In fact, it has been shown that iron uptake by this mechanism is entirely independent of transferrin. Sturrock et al demonstrated iron uptake in HeLa cells in the presence of inhibitors of receptor-mediated endocytosis and at a pH of 5.5 at which transferrin binds iron poorly (43). It is thought that the uptake occurs between low molecular weight iron-organic anion chelates by a membrane-based system present on a variety of cultured cell types (44). The iron substrate is believed to be the more soluble ferrous form, possibly following cell surface reduction by a mammalian ferrireductase similar to that in bacteria and fungi (45). Using such iron chelates (^{59}Fe-nitriloacetate, ^{59}Fe-citrate, and ^{59}Fe-Tricine ascorbate), Sturrock et al demonstrated that uptake in HeLa cells was time- and concentration-dependent, saturable, and subject to competitive inhibition by other divalent metals. In K562 cells metals such as Co, Ni, and Mn permeated at rates similar to iron (46). These results suggested that the transport mechanism consisted of a carrier for several divalent metals including iron.

It is apparent from studies on the regulation of the non-transferrin system that its purpose is widely divergent from that of the transferrin pathway. Some transferrin independent pathways are unique in that they do not necessarily exist to fulfill cellular iron needs. In cultured HeLa cells and fibroblasts, Kaplan et al produced iron uptake that was independent of changes in cell growth rate, induction of DNA synthesis and cell division, or cellular iron needs. What did increase transport activity, however, was mere exposure of the cells to either ferrous or ferric iron. Kaplan found that pre-incubation of

cultured cell lines with inorganic iron (ferric citrate) actually enhanced uptake of subsequent radioactive iron (44). Based on these results it was proposed that extracellular iron was capable of catalizing the appearance and activity of the membrane iron transporters responsible for its uptake. Parkes et al similarly demonstrated a positive correlation between cellular iron content in cultured rat myocardiocytes and the rate of non-transferrin-bound iron uptake (47). As it was then proposed, the function of this iron transport pathway seems to be the clearance of non-Tf iron from plasma—a physiologic 'salvage pathway.'

A candidate iron transporter gene has recently been identified. The gene, initially called Nramp2, belongs to a family of ubiquitous trans-membrane transporters purportedly involved in dietary iron absorption. Nramp2 was found to co-segregate with a phenotype of homozygous mk/mk mice that have microcytic, hypochromic anemia due to severe defects in intestinal iron absorption (48). The relationship between Nramp2, now called DMT1 (divalent metal transporter 1), and the defective HFE gene in hereditary hemochromatosis is poorly characterized. Whatever the primary defect in intestinal iron transport, a recent study on regulation of DMT1 expression makes it doubtful that the same defect is responsible for the parenchymal overload in hemochromatosis and transfusional iron overload. In HFE knockout mice, hepatic DMT1 expression was not elevated despite increased expression of this transporter in duodenal cells (24). Also, it appears that DMT1 expression is appropriately regulated by cellular iron status and therefore would not continue iron transport into iron-loaded cells. Gunshin et al found that in iron deficient mice, DMT1 expression in the duodenum was suitably increased (49).

Iron Overload States

Clearly in states of iron overload, though, iron enters parenchymal cells in amounts sufficient to cause multi-organ failure. Liver iron content in patients with hereditary hemochromatosis is between 50-100X normal. The 20-40 g of total body iron accumulated in patients with hemochromatosis far exceeds normal levels of approximately 3 g (15). Iron content in the liver of a double organ transplant hemochromatotic was 28.1 mg iron/gm dry weight (47). Normal males, in comparison, have 0.17 mg iron/gm dry weight (50). Untreated thalassemia patients accumulate toxic amounts of iron by age ten and lethal amounts by adolescence (51). Two questions arise, then, when considering parenchymal iron burden in iron overload diseases. What is the mechanism of transport into parenchymal cells? And, is it physiologic or does it reflect a defect in regulation similar to the primary defect present in intestinal iron absorption?

It was initially speculated that a defect in transferrin receptor was responsible for the inappropriate iron uptake by parenchymal cells in states of iron overload. Gatter's discovery that the selective organ distribution of TfR expression paralleled that of tissue damage in hereditary hemochromatosis, lead to his proposition that high levels of transferrin receptor expression might be responsible for increased iron uptake in the liver and pancreas (54). Further studies on TfR expression revealed that this association was not causal. Ward et al investigated characteristics of TfR regulation in patients with hereditary hemochromatosis to uncover whether any disregulation of this receptor was responsible for the massive tissue iron accumulation. Using cultured fibroblasts and mitogen-stimulated lymphs from HH patients, it was shown that transferrin receptor

number and affinity, as well as the ability to down-regulate was similar to that in cells from normal controls (53).

Despite the appropriate down-regulation of the transferrin receptor, parenchymal iron overload still occurs. It appears that non-transferrin iron uptake, which actually up-regulates in conditions of cellular iron loading, is the operative pathway. Melanoma cells preincubated with ferric ammonium citrate have shown increased non-receptor mediated uptake of iron from transferrin and a decrease in receptor-mediated uptake (54). Secondary to the saturation of transferrin, high levels of non-transferrin iron are present in the plasma of hemochromatotics. Whereas normals have between 0.6-1 uM non-transferrin bound iron, levels approach 5-15 uM in hereditary hemochromatosis (55). Hepatic clearance of this form of iron appears to be both efficient and rapid, suggesting that non-transferrin bound iron could be the direct cause of toxic iron burden in the liver. Brissot et al, using a rat liver perfusion system demonstrated that hepatic uptake of 1uM non-transferrin ^{55}Fe from human serum had a single-pass extraction efficiency of 58-85%. The K_m of uptake, at 14-20 uM iron, interestingly approximated the levels of non-transferrin bound iron in states of overload (56). Another study in Tf iron-saturated rats and found that more than 80% of subsequently injected radiolabelled iron was cleared from the plasma by 30 seconds. PAGE analysis revealed that this iron was in a non-transferrin associated form (57). These results are compatible with the knowledge that intestinally absorbed iron in HH patients is efficiently removed by the liver before entering systemic circulation (58).

The predicament of parenchymal iron loading is clear. Appropriate down-regulation of transferrin-mediated pathways is insufficient to avoid overload.

Simultaneously, the uptake of non-transferrin bound iron is functioning maximally, causing a great deal of cellular toxicity. Unless plasma iron levels are reduced this pattern will continue unchecked. How then can excess iron be eliminated from the body? Absorption may not be the best site for effecting change because the exact mechanism of normal and abnormal iron uptake is unclear. Chelators can increase urinary iron excretion, but the inconveniences of this mode of therapy have previously been illustrated. The epidermis, which is already responsible for nearly 25% of daily body iron excretion, has not yet been considered a site for therapeutic intervention. Interestingly, though, this route of iron elimination is already slightly enhanced in patients with HH. Increased amounts of iron are present in the basal epidermal layer of patients with hemochromatosis (59). Also, measures of skin iron content by X-ray spectrometry have been shown to correlate well with body iron stores (60). These findings suggest that excessive iron can be delivered to keratinocytes and that keratinocytes are capable of augmenting their iron stores. Therefore, the goal of artificially enhancing epidermal iron losses is consistent with a pre-existing physiologic response.

Iron in the epidermis

Information about iron in the epidermis is somewhat limited. The specificity of epidermal iron distribution most likely reflects the selective expression of the transferrin receptor in the basal epidermis (61). Indeed, proton microprobe analysis reveals that iron is more abundant in the basal layers of the epidermis (62). Dermally injected ^{59}Fe demonstrates a predominance of iron in the basal layer of the stratum malpiphii that

decreased toward the stratum granulosum (4). Various methods have been employed to measure epidermal iron content. Estimates range from approximately 0.03 to 0.24 mg iron/gram dry weight in normal skin (2, 63). In normal necropsy specimens, the total iron content of epidermal skin was determined to be 22.5 ± 17.8 mg (64); a small amount relative to the liver iron stores of 2.2 mg per gram (Mayo Labs upper limit of normal).

There are two potential routes for epidermal iron loss—desquamation and perspiration. Initially, there was much controversy about the relative contribution of sweat to body iron loss. Numerous studies concluded that the amount of iron lost in sweat approached several mg per day (65, 66, 67). Sweating was even implicated as an etiologic factor in iron deficiency in tropical climates (68). Such diverse results represent the inherent difficulties of measuring iron loss because of the small quantities measured, methodological error, and environmental contamination with iron (69). Present understanding of body iron balance also makes the results of those studies dubious at best. A convincing study by Green et al concluded that perspiration was an insignificant source of total body iron loss. "Seattle whites" at desk jobs lost 0.95 mg total iron per day compared to steam laundry workers in Durban, South Africa who, despite conditions of high temperature and humidity, still lost only 1.02 mg iron per day (64). A more recent estimate of cell-free sweat iron loss was calculated to be approximately 0.01 mg/day—a trivial portion of the 1-2 mg total lost daily (7).

It is now appreciated that the major source of epidermal iron loss results from desquamation. These losses actually represent a significant fraction, about 20-25%, of daily total iron losses, making the skin an important participant in iron homeostasis. Weintraub was one of the first to propose that the skin 'acts as an active excretory organ

for iron.' He found that whole body iron loss following intravenous injection of ^{59}Fe citrate was 3-fold greater than could be accounted for by urine and stool losses alone. The remainder, he proposed, was lost through desquamation (4). The amount of skin iron loss has been estimated by calculations based on various parameters, including epidermal turnover time and skin iron content. Cellular turnover of the skin—the time it takes for a basal cell to traverse the epidermis and desquamate—is between 26-28 days. In healthy individuals, up to 1.0 g of normal epidermis is desquamated per day (70, 71). From a recent measurement of skin iron content, 0.24 mg iron/gm dry weight (63), then, one could predict that 0.24 mg iron would be lost daily via desquamation. Using estimates of total epidermal iron content of between 3-14 mg and as 22.5+/-17.8 mg, Bothwell hypothesized that daily skin iron loss ranged from 0.2 mg to 1.2 mg (15).

An alternative approach to estimating desquamative iron losses is to assume that daily skin iron loss represents a steady state and that the amount of iron entering the skin equals the amount exiting. Green et al intravenously injected ^{59}Fe in to patients 16-24 hours before they were scheduled to undergo operations in which skin would be removed. They found that at normal transferrin saturations, skin iron uptake from plasma was between 0.2 and 0.3 mg per day (64).

In disrupted metabolic states skin iron uptake and losses diverge from normal. One can imagine that the primary ways by which to increase skin iron loss would be either to increase cellular turnover time or to increase skin iron content. Two diseases—psoriasis and hemochromatosis— reproduce these conditions, respectively. In hyperproliferative states like psoriasis where turnover time is increased 4-fold, skin iron losses increase, possibly contributing to iron deficiency in these patients (72). The

question of iron levels in the skin of hemochromatotics has not been satisfactorily resolved. It is known that the 'bronze' skin discoloration is due to the accumulation of melanin rather than epidermal iron or hemosiderin (73). Pathologic iron deposition, however, does occur in the skin, mainly in the basal layers (74). Some studies, though, do imply that there is an augmentation of epidermal iron uptake in situations of iron overload. Green et al found that skin iron uptake increased linearly with a rise in transferrin saturation—from the normal 0.2-0.3 mg/day to 0.6-0.7 mg/day (64). In another study, intravenous radioiron injections produced positive Prussian blue staining of the epidermis, indicating that intracellular iron existed in excess of physiologic cellular needs (4). The correlation of skin iron concentration and parenchymal tissue iron in iron overload has already been mentioned. One would assume, then, that HH individuals have higher than normal skin iron losses. Despite the belief that no excretory mechanism exists for iron, data on iron overload patients suggests that late in the disease a balance between absorption and excretion is approached. Daily iron losses increase from the normal 1 mg/day to 1.5 mg/day in these patients, particularly due to increasing skin iron losses (15). This indicates that a vector of transfer exists between plasma and skin and adds support to a design whereby skin could be exploited as an organ of iron excretion.

Are there ways to further shift the burden of iron load to keratinocytes? In most if not all cell types the transferrin-mediated uptake is down regulated in iron overload. Non-transferrin bound iron uptake is already optimized and still not enough iron is lost through the skin. Recently, a candidate for an alternate route of iron loading has been identified. The discovery, as it turns out, was serendipitous. A group of hematologists, aware that iron transport is calcium sensitive, were studying the effects of calcium

channel blockers on iron uptake in erythroid cells. Specifically they were looking for inhibition of iron uptake in the presence of calcium channel blockers. The surprising result was a 3 to 4 fold increase in uptake in the nifedipine-exposed cells. The effect only occurred when the nifedipine was photodegraded; light-shielded nifedipine was ineffectual. They localized activity to the major photodegradation product of nifedipine—a fully aromatic nitrosopyridipine derivative. The nitroso-derivative produced a temperature sensitive, bi-directional change in uptake of non-transferrin iron only; no effects were seen with transferrin bound iron. Iron uptake by the nitroso-derivative occurred at a different pH optimum and in the presence of known inhibitors suggesting that these effects were independent of the established mechanisms of non-Tf iron transport. It was proposed that the PDN derivative represented a 'mobile iron ionophore' (75).

The effects of photodegraded nifedipine are not limited to iron transport. Luttrop et al were attempting to enhance gallium uptake in tumor cells for improved radiographic imaging. Because gallium is an iron analogue, they predicted that PDN might similarly mediate its cellular uptake. Using 25 uM photodegraded nifedipine, they achieved up to a 1000-fold increase in Ga-67 uptake. The effect was independent of transferrin and its receptor. As PDN did not alter the lipophylicity of Ga-67 citrate, they concluded that the mechanism of PDN-mediated gallium uptake does not involve the formation of a lipophyllic complex or carrier that diffuses across membranes (76). This implies a mechanism independent of DMT1—a known iron carrier.

Nifedipine is the prototype of the dihydropyridine class of calcium channel antagonists. It is used commonly to treat hypertension and angina pectoris. Nifedipine's

photodegradable properties have been known for a long time. In 1931 a qualitiative observation was made that a specific class of dihyropyridines was sensitive to light. Another early paper cites the 366 mu wave length which catalyzes a reduction of the nitro- group to produce an aromatizing dehydrogenation (77). Different light sources yield different reaction products. Exposure of nifedipine to UV light produces a *nitro*phenylpyridine product. Daylight results in the predominance of a *nitroso*phenylpyridine product (78).

The above reactions proceed quickly because dihydropyridines are remarkably light-sensitive, with half lives in the range of minutes (79). Photodegradation products of nifedipine have even been isolated from hospital prescriptions (80). This characteristic makes nifedipine a potentially safe therapeutic agent. In the photodegraded form it has only minimal calcium channel blocking activity suggesting that the pharmacological effects of nifedipine are different from those of its derivatives. Several recent studies have documented that nifedipine-induced reduction of calcium current amplitude can be reversed within milliseconds after a brief light flash (81, 82).

Hypothesis

The founding idea of this project is that the normal process of epidermal desquamation can be harnessed to eliminate toxic chemicals from the body. The presumption is that keratinocytes are capable of accumulating and retaining iron and that this iron load would be lost from the body by desquamation.

Our hypothesis is that photodegraded nifedipine represents an effective way to pharmacologically increase the iron content of human keratinocytes. Iron uptake and total iron content in keratinocytes exposed to PDN *in vitro* will be measured. We will investigate the effect of time of PDN exposure, dose of iron, and state of differentiation on the characteristics of uptake.

Methods

Cell Culture

Human neonatal foreskins were obtained from the Yale Children's Hospital. Tissue was washed twice and incubated overnight at 4°C in dispase. The following day, the tissue was removed from the incubator, the epidermis was peeled from dermis and then incubated at room temperature for 10 minutes. The epidermis was incubated at 37°C for 15 minutes in 3 mL of trypsin + EDTA. 4 mL STI was added to stop the reaction. Cells were counted, centrifuged, then resuspended in cMCDB and seeded at approximately 5 x 10^6. Cells were split after reaching 50-70% confluence. We used keratinocytes at 3rd, 4th, or 5th passage when grown to a near confluent monolayer in 6-well plates. (83, 84)

Photodegradation of Nifedipine:

Nifedipine (Sigma chemical Co., St. Louis, MO) was dissolved in 1 mL ethanol for a concentration of 10 mmol/L. The sample was shielded from light and stored at -4°C until intended photodegradation was conducted (76). To photodegrade the nifedipine, the sample was exposed to direct outdoor daylight in a clear 1.5 ml polypropylene microfuge tube for exactly one hour. Adequacy of photodegradation was confirmed by thin-layer chromatography (TLC) on silica gel using a hexane:chloroform:acetone solvent in a 50:30:20 ratio (75). The TLC was then photographed on a UV light box using Polaroid film.

Preparation of Iron Incubation Solution

An incubation medium with a 15 uM concentration of iron was used in all experiments unless otherwise stated. 1 uM of the above medium was radioactive iron, $^{59}FeCl_3$ (Dupont NEN, Fe-59 chloride). The other 14 uM was made up with $^{56}FeCl_3$ (Fisher Scientific Co., Fair Lawn, N.J.). The appropriate volumes of Fe-59 and Fe-56 were combined in a 15 mL centrifuge tube. A molar excess of Tricine ascorbate (10 mM tricine, 0.3 mM ascorbate) made in Hank's balanced salt solution (HBSS) was added to the iron. Tricine was selected as a nonmetabolized amino acid that binds to, and effectively solubilizes, both ferrous and ferric iron (56). Ascorbate is an important coordinator of ferrous iron in plasma (85). The HBSS, pH 7.2-7.4, contained 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 3.7 g/L $NaHCO_3$ (76). The iron/tricine ascorbate solution and a separate 6 ml of HBSS in a 15 mL centrifuge tube was allowed to equilibrate uncapped at 37°C in a 4% CO_2 incubator for 30 minutes prior to use.

^{59}Fe Uptake

The following was adapted from Luttrop et al (76). After growth of keratinocytes in 6-well plates to near confluence, monolayers were washed and then preincubated for 2 hours at 37°C in 2 ml serum-free Dulbecco's Modified Eagle medium (DMEM) (GIBCO Laboratories, Grand Island, NY) to deplete cells of transferrin. At the end of 2 hours, the preincubation medium was removed and replaced with 1 mL per well of incubation solution containing 15 uM iron (76). The incubation solution for the experimental group contained 25 uM photodegraded nifedipine (PDN); controls received an equal

concentration of ethanol. Cells were incubated in a 4% CO_2 incubator at 37°C for varying intervals of time, depending on the experiment.

Cell Harvest

The following protocol was adapted from Luttrop et al (76). Following incubation with ^{59}Fe, flasks were immediately placed on ice. The incubation medium was removed by aspiration with a Pasteur pipette attached to water suction. Cell monolayers were washed once with ice-cold calcium magnesium free phosphate-buffered saline (CMF PBS), then twice with CMF PBS prewarmed to 37°C. CMF PBS was removed. To obtain a cell suspension, monolayers were trypsinized with 700 uL 0.05% trypsin + EDTA at 37°C for 3-4 minutes. The cells and trypsin were collected and carefully layered over a 200 uL column of dibutylphthalate:paraffin oil (8.5:1.5) in 1.5 mL tubes. To separate the cells from contaminating extracellular radioactivity, the tubes were centrifuged at 11,000 rpms for 2 minutes. The supernatant was then aspirated with a Pasteur pipette attached to water suction. Using a microfuge tube clipper, the bottom of the microfuge tube containing the cell pellet was clipped into a counting vial containing 900 uL solution of 200 mmol/L NaOH and 1% sodium dodecyl sulfate. The amount of radioactivity in the cell pellets was determined by gamma counting (CompuGamma 1282, PerkinElmer Wallac, Inc.)

Protein Assay

Protein assays of the solubilized cell samples were performed by formation of the cuprous bicinchoninic acid complex, and quantified according to absorption on a

spectrophotometric microtiter plate reader (Dynatech Laboratories). The method and reagents used are supplied in a kit (Pierce Chemical Co., Rockford, IL). Bovine serum albumin was used as a standard of reference.

Stratified Cell Cultures

Human keratinocytes were seeded into 6-well plates (9 cm^2/well) and grown in cMCDB medium until confluent. At confluence, the medium was changed to DMEM/ F12 + 10% fetal calf serum (FCS) to allow cells to stratify. This was a modification of the method in Zhou et al (83). One week after the medium change, the stratified cell layers were separated from the underlying basal cells as follows. The DMEM growth medium was aspirated and cells were washed twice with CMFPBS. Cells were then incubated at 37°C in a CO_2 incubator for 10 minutes in 2 ml of versene (GIBCO, Grand Island, NY). Immediately following incubation, the stratified cell layer was gently separated from the basal layer with forceps (83). The sheet of stratified cells was then washed twice in a separate dish with PBS + 0.5 mM $CaCl_2$. to saturate any remaining versene. Each stratified layer was then laid in the well of a 6-well plate containing 2 ml of DMEM/F12 + 10% FCS and incubated at 37°C overnight. The versene left in the wells containing the intact basal cells was aspirated. These basal cells were washed 3X with PBS + 0.5 mM $CaCl_2$ then incubated overnight at 37°C in 2 mL cMCDB to prevent differentiation.

On the day of the experiment growth medium was removed. The stratified, free-floating suprabasal, and basal cells layers were preincubated in regular DMEM for 2 hours. Iron uptake studies with 15 uM iron, +/- 25 uM PDN were conducted at 37°C for

20 min as previously described. The collection of the cells differed slightly. Basal cells were trypsinized in 0.05% T+E for 1 min, spun through oil, and gamma counted. Stratified cell layers were gently scraped from the wells with a rubber policeman and washed in CMF PBS three times. The suprabasal cells were removed intact to vials containing 900 ul of NaOH/SDS. Gamma counting and protein assay were performed on these two cell types.

Statistical Methods

In all experiments, standard deviation was calculated for each experimental and control group. This variance is represented as vertical error bars on all displayed graphs.

Results

Thin-layer chromatography

A 10 mM solution of nifedipine in absolute ethanol was divided into 3 equal aliquots in 1.5 ml plastic microfuge tubes. One sample was shielded from light. The other two were exposed to outdoor daylight for time intervals of 30 and 60 minutes. These solutions were run on a silica gel using a hexane:chloroform:acetone solvent in a 50:30:20 ratio (75). Thin layer chromatography showed the disappearance of the original substance and the appearance of a major photodegradation product with increasing duration of light exposure. This product has previously been identified as a fully aromatic nitrosopyridine derivative of nifedipine (75,76). It is the major product of daylight-induced degradation of nifedipine (78). Other minor degradation products have also been identified but could not be detected here because of the insensitivity of the employed method. The RF value of nifedipine is 0.68; that of the photodegradation product is 0.76.

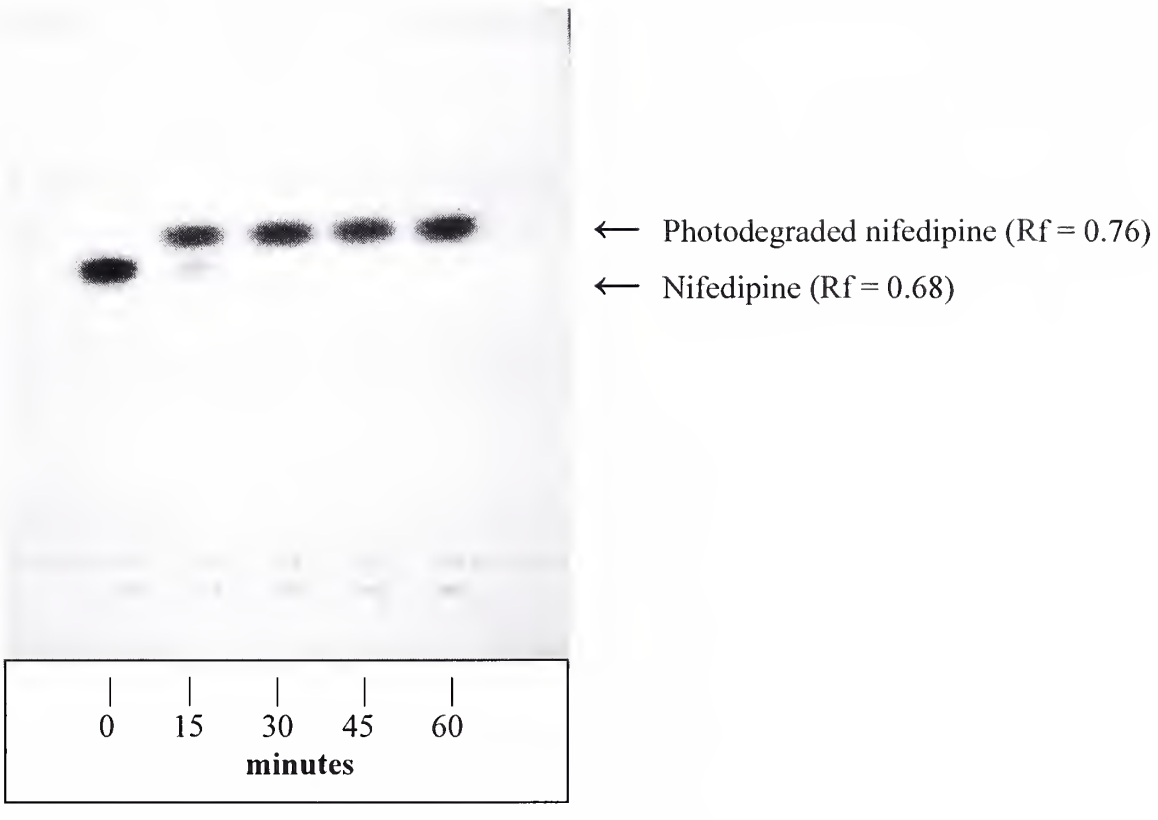

Fig 2. Thin Layer Chromatography of nifedipine exposed to daylight. A 10 mM solution of nifedipine was exposed to outdoor daylight for variable intervals of time and examined by thin-layer chromatography. This experiment is representative of experiments performed three times.

Iron Uptake

The effect of photodegraded nifedipine on free iron uptake in erythroid cells (75) and of gallium uptake in CHO and mouse 3T3 lines (76) has already been documented. In these cell types degree of metal uptake varied considerably. Erythroid cells demonstrated approximately 3 to 4-fold increase in iron uptake when exposed to PDN, whereas CHO and mouse 3T3 lines boasted gallium uptake of 1000-fold. We demonstrated that PDN is capable of stimulating iron uptake in normal human

keratinocytes. After a 30 minute incubation with 15 uM solution of ^{59}Fe in the presence of 25 uM PDN, proliferating monolayers of keratinocytes produced an 18-fold increase in iron uptake. This was significantly different from non-transferrin bound iron uptake in control cells incubated in equivalent concentrations of ethanol. The experiment was repeated twice.

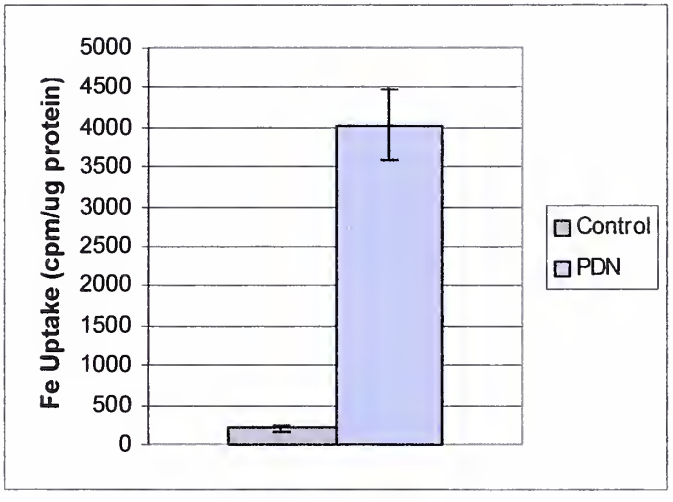

Fig 3. Iron uptake in basal keratinocytes exposed to photodegraded nifedipine. Normal human keratinocytes were treated for 30 minutes with 25 uM PDN or ethanol and 15 uM ^{59}Fe, as described in methods. The PDN-exposed group demonstrated a 20-fold increase in iron uptake versus controls. Results are mean ± S.D. of 6 samples. This data was combined from two identical experiments.

Time Course of Iron Uptake

There is a time-dependent cellular uptake of iron with exposure to photodegraded nifedipine. Preconfluent proliferating keratinocytes were incubated in 15 uM iron with or

without 25 uM PDN and harvested at 1, 5, 15, 30, 60, 120 and 180 minutes. Uptake occurred rapidly. By one minute, keratinocytes incubated in the presence of PDN demonstrated a 6.7-fold increase in iron uptake above controls. Longer periods of incubation revealed a linear increase in iron uptake that eventually plateaued. The plateau was reached between 60-120 minutes. The most significant increase in PDN-mediated iron uptake above that in controls (presumably non-transferrin iron uptake) occurred at 30 minutes where uptake was approximately 87-fold greater than controls.

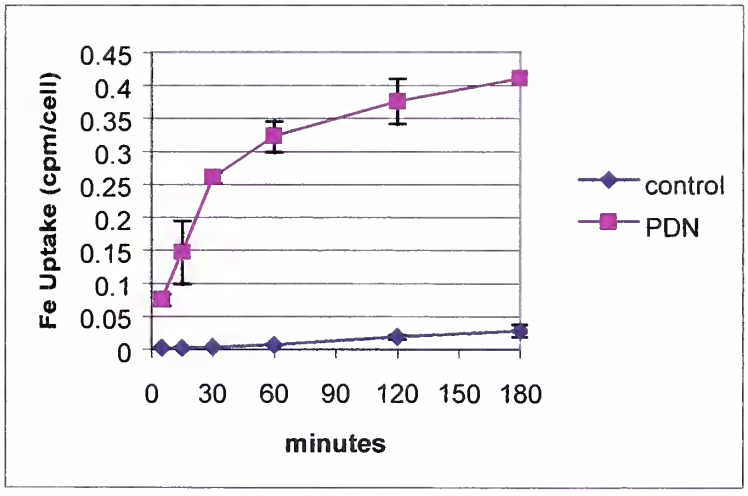

Fig 4. Time course of iron uptake in PDN and control keratinocytes. Keratinocyte monolayers were incubated at 37°C with 15 uM Fe in the presence or absence of 25 uM PDN. Each data point represents the mean of three samples ± S.D. This experiment was repeated three times.

Dose Response

Preconfluent monolayers of keratinocytes were incubated for 20 minutes at 37°C with either 1 uM, 5 uM, 15 uM, 20 uM, or 30 uM concentrations of iron. 25 uM PDN was added to the incubation solution in experimental groups. Controls received equal concentration of ethanol. Cells were harvested and iron uptake as represented by umole iron/cell was calculated from measured cpm values. There is a clear dose-response relationship between iron concentration in the incubation medium and cellular iron uptake. At concentrations as low as 1 uM, significant uptake of iron was evident in the PDN-exposed cells. These cells took up 77–fold more iron than controls. Though the greatest amount of uptake occurred at the highest iron concentration tested (30 uM), the largest difference between PDN and control cells occurred between 1-15 uM and peaked at 5 uM. At this point experimental groups demonstrated a 102-fold difference in uptake from controls.

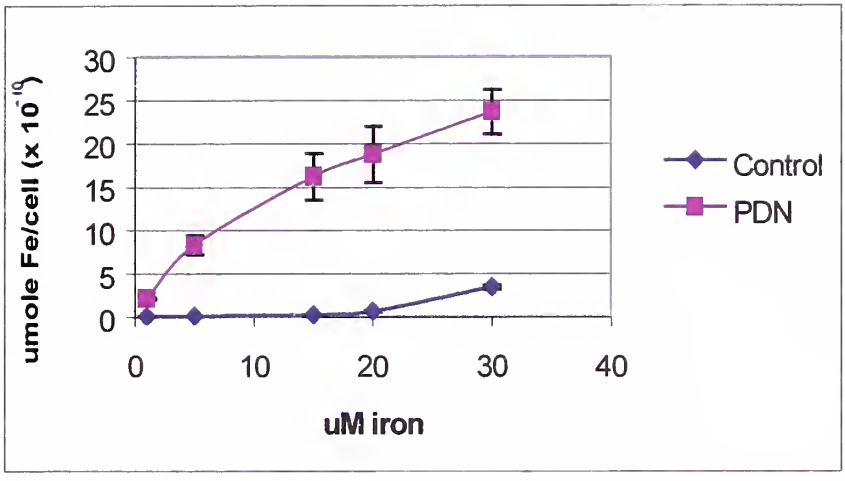

Fig 5. Response of iron dose on uptake in keratinocytes. Preconfluent monolayers of keratinocytes were exposed to varying concentrations of ^{59}Fe for 20 minutes at 37°C. Each data point represents the mean of 3 samples ± S.D. This experiment is representative of experiments performed twice.

Effect of pH on uptake in stratified cells

The effect of PDN on iron uptake raises two possibilities. Either PDN augments the existing non-transferrin pathway or it functions independently. Savigni et al demonstrated that PDN increased iron uptake in the presence of potent inhibitors of non-transferrin uptake. They also found that PDN and non-transferrin mediated iron uptake in erythroid cells was optimal at different pH levels (75). We wished to examine whether a similar disparity occurred in keratinocytes. An isotonic incubation solution made of 20 mM PIPES buffer in 150 mM NaCl was titrated with 1 normal NaOH to give solutions of pH 6.1, pH 6.5, pH 7.0, and pH 7.5. Stratified keratinocytes were incubated with 15 uM iron with or without 25 uM PDN in the varied pH incubation solutions for 20 minutes. Iron uptake in the PDN exposed cells was markedly increased over that of controls at all pH values tested. There is a clear difference in pH optimum between the PDN and

control groups. PDN-mediated iron uptake occurred maximally at pH 7.5 or greater. Alternately, iron uptake by keratinocytes in the absence of PDN peaked distinctly at pH 6.5.

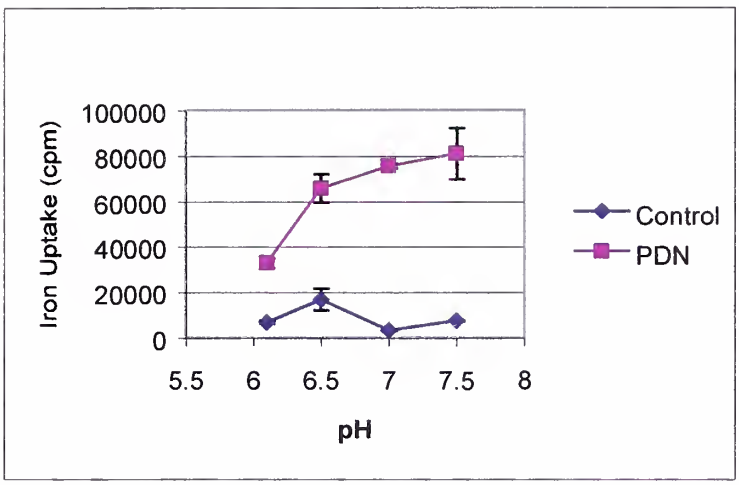

Fig. 6. Effect of pH on iron uptake in keratinocytes. Cells were incubated with 15 uM Fe for 20 minutes at 37°C in isotonic NaCl buffered at the indicated pH values with 20 mM PIPES. Experimental cells had 25 uM PDN added to the medium. Each data point is the mean of 3 samples ± S.D. Note the clear difference in peak iron uptake between PDN-exposed cells and controls. This experiment was performed once.

Iron Uptake in stratified cell layers: Stratified vs. Basal vs. Suprabasal

We sought to uncover whether differentiation state would affect cellular iron uptake. As described in methods, we conducted iron uptake experiments in basal, suprabasal and stratified cell layers. Cells were incubated at 37°C for 20 minutes in 15 uM iron either with 25 uM PDN (experimental group) or 25 uM ethanol (controls).

There was significantly increased iron uptake in all PDN-exposed cells. The greatest amount of uptake in cpm/ug total protein occurred in the stratified cells. In these cells, a 16.4-fold difference in iron uptake over controls is apparent. PDN-incubated suprabasal cells did not show as great a difference, 3.7-fold, compared to controls.

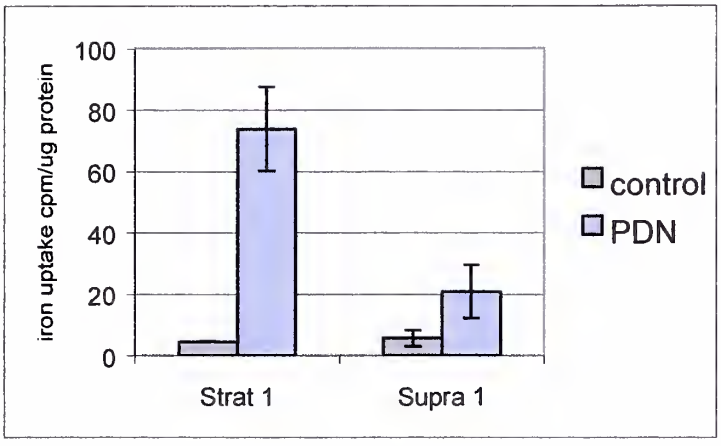

Fig. 7. Effect of cellular differentiation on iron uptake. Intact, stratified keratinocyte cell layers (Strat 1) and detached suprabasal, stratified cell layers (Supra 1) were incubated with 15 uM Fe for 20 minutes at 37°C in the presence of absence of 25 uM PDN. The results reveal a greater difference in uptake between PDN-exposed cells and controls in the intact, stratified group. Each bar represents the mean of 3 samples ± S.D. This experiment was performed twice.

Iron Accumulation

The experiments studying iron uptake demonstrate that uptake occurred during a single, brief exposure to PDN. We were interested to see whether repeated exposures of cells to iron in the presence of PDN would actually alter cellular iron content. Twenty two x 60 mm dishes of normal human keratinocytes were grown to confluence and then

allowed to stratify in DMEM/F12 + 10% FCS for one week (84). On the first day of experiments, 6 dishes were sacrificed for time zero protein assay and iron content. The remaining 16 dishes were incubated at 37°C for one hour with 20 uM ^{59}Fe. In half of the dishes, the incubation solution contained 25 uM PDN, the others consisted of 25 uM ethanol. At the end of the 1 hour incubation, cells were washed, replaced in growth medium (cMCDB + 1.4 mM Ca^{+2}), and returned to the incubator. This procedure was repeated daily for a total of five days. After the final iron incubation, cellular iron content was measured by inductively coupled plasma mass spectrometry. PDN-treated cells showed a 3-fold increase in total iron content. Iron content in these cells averaged 403.4 ± 22.4 ug iron/g dry weight compared to controls whose average iron content was 147.6 ± 10.7 ug iron/g dry weight. Jing Zhou, Ph.D. performed this experiment.

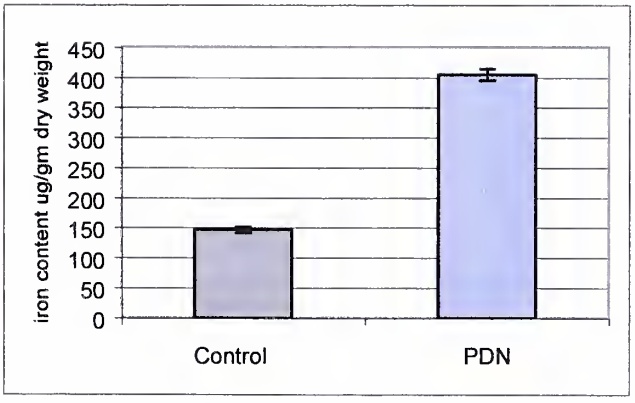

Fig. 8. Accumulation of iron in keratinocytes exposed to PDN. Stratified keratinocyte cell layers were incubated with 20 uM Fe with or without 25 uM PDN for 1 hour at 37°C. This procedure was repeated daily for a total of five days. Cells were sent for analysis of iron content. Each bar represents the mean of five samples ± S.D.

Iron Retention

Keratinocytes also demonstrated the ability to retain iron after loading in the presence of PDN. Confluent, stratified keratinocytes were treated for 1 hour in 20 uM Fe-tricine ascorbate with or without 25 uM PDN. Cells were washed and returned to standard medium (DMEM/F-12 with 10% FCS) for 24 hours. This procedure was repeated daily on 4 successive days. Epithelia were maintained in standard medium an additional 4 days then sent for analysis of iron content by inductively coupled plasma mass spectrometry. PDN-treated samples showed a 3-fold increase in total iron content that persisted through the washout period. This experiment was performed by Jing Zhou, Ph.D.

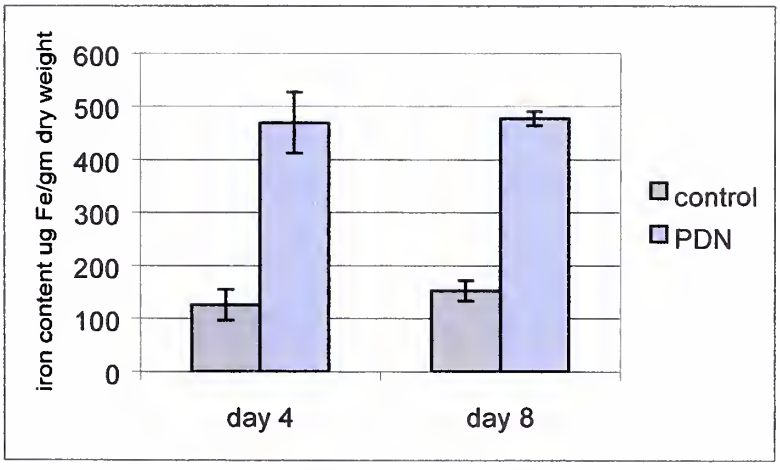

Fig. 9. Retention of iron after washout in keratinocytes exposed to photodegraded nifedipine. On days 0-4 cultures were pulsed once daily with iron (20 uM) and PDN (25 uM) or ethanol (controls). Iron content was measured as described in methods. Accumulated iron persisted 4 days beyond the last PDN treatment.

Discussion

Our results demonstrate the powerful effect of PDN in stimulating non-transferrin bound iron uptake by keratinocytes in vitro. The effect of PDN on free iron uptake in erythroid cells (75) and of gallium uptake in CHO and mouse 3T3 lines (76) has already been documented. The light dependence of the PDN-mediated iron uptake has already been shown by Savigni and was not tested here. Light-protected nifedipine does not stimulate Fe (II) uptake in erythroid cells (75). Kathryn Morton has crystallized the nitroso-derivative (nitrosopine) and shown it to be the active derivative (Morton, personal communication).

We found that the magnitude of PDN-mediated iron uptake surpassed uptake in control cells by nearly 18-fold during a 30 minute exposure. This degree of augmentation exceeds the 3-4 fold increase of PDN-mediated iron uptake in erythroid cells found by Savigni (75). The variability in iron uptake achievable by PDN between our results and those of Savigni most likely reflects the specific parameters of the cell types studied. If PDN serves as ion channel for diffusion as Savigni proposes, then cells with higher iron content, such as erythroid cells, might not accumulate as much iron as keratinocytes. As for the range of results produced between experiments in keratinocytes, the cause is unclear. It is possible that subtle differences in pH of the incubation medium from one experiment to the next are responsible. Our incubation buffer (HBSS) was bicarbonate-based and thus differences in time out of the incubator may have promoted escape of CO_2. Experiments using PIPES buffer (non-bicarbonate based) demonstrate the sensitivity of iron uptake to changes in pH.

The results of the uptake experiment were an important first step. However, the pivotal question to our underlying goal of dermatoremediation of iron overload is whether we can alter the iron content of keratinocytes. We achieved a net iron accumulation over a five-day time course by exposing cells to PDN and iron simultaneously. More importantly, the 3-fold augmentation of iron content persisted through the washout period. This suggests that iron taken up by keratinocytes *in vitro* can be retained. Using erythroid cells, Savigni et al demonstrated that efflux of ^{59}Fe from reticulocytes after PDN-stimulated uptake was negligible over several hours, unless PDN and desferrioximine were present in the culture medium (75). Other studies of cellular iron transport support the notion that iron can be retained after iron loading. Radioactive iron injections in Tf iron-saturated mice persisted in liver and pancreas 14 days after injection (57). Another study in myocardial rat cells there was no spontaneous release of radioactive iron from cells without the presence of an iron chelator (32). Current understanding of intracellular iron flux helps explain this phenomenon. It is known that iron first passes through a transient, chelatable pool of free iron before being bound and stored by ferritin. The transit time through this pool is thought to be about one to two hours (46, 86). After being bound by ferritin, iron is less efficiently liberated from the cell. Even chelators can access only a fraction of this storage iron. One could then predict that if enough ferritin existed to store newly delivered iron, that iron would be unlikely to efflux spontaneously the cells.

To increase the amount of iron that could potentially be lost through the skin by desquamation of iron-loaded keratinocytes, one must find a way to maximize it's accumulation. Repeated exposures of keratinocytes to iron and PDN might increase

storage capacity. High levels of regulatory or chelatable intracellular iron are known to induce ferritin synthesis by stabilizing its mRNA (87). In this way it is possible that iron could be stored in a greater amounts without exposing the cells to a sudden overload of the more toxic, chelatable form of iron.

Other parameters of PDN-mediated pathway were investigated to optimize iron uptake in keratinocytes. These experiments revealed the time- and dose-dependent manner by which PDN-mediated iron uptake occurs. The characteristics of our time and dose curves closely resemble those achieved by Savigini et al in studies of PDN-mediated iron uptake in erythroid cells (75) and Morton's findings of ^{67}Ga uptake in CHO and mouse 3T3 lines (76). The latter group found that maximal uptake of Ga occurred when cells were incubated for 30 minutes with 25 uM nifedipine that had been photodegraded using fluorescent light (76). Our maximal uptake in terms of the greatest difference between experimental (PDN) and control groups similarly occurred at 30 minutes. At this time point uptake of ^{59}Fe was as much as 87-fold greater than basal levels. It is also important to note that significant uptake developed rapidly. After one minute (data not shown), there was already a 6.7-fold difference in the presence of PDN. Interestingly, basal iron transport (non-transferrin iron transport) followed a slightly different time-response curve. We expanded the control curve in four of the representative experiments and found that there was a lag time to its development (data not shown). Increased iron uptake occurred around 30 minutes. This finding is somewhat discrepant with other studies of non-transferrin bound iron uptake. However, these studies were conducted in different cell types--fibroblasts (44) and HeLa cells (43) and at lower ^{59}Fe concentrations than the 15 uM that we used. If it is real, the difference we detected in the shape of the

time-curves for non-transferrin and PDN-mediated iron transport could represent differences in the kinetics or their mechanisms of transport; for example, carrier-mediated vs. diffusion.

Our time curve plateaued between 60-120 minutes. The plateau could represent one of two phenomena. Either internal storage mechanisms for iron become saturated or the rate of iron influx equals rate of iron efflux or leakage. Our experiments demonstrating iron retention over a period of days makes it unlikely that significant leakage of iron occurs during the 1-2 hours investigated during the time course.

Not only did PDN-mediated iron uptake occur expeditiously, but it also was very sensitive to increases in iron concentration in the extracellular medium. The dose-response curve in the PDN-stimulated cells developed at concentrations as low as 1 uM iron (lesser concentrations not tested) and continued to show significant uptake to 30 uM where a plateau was just beginning. We were particularly interested in the amount of iron uptake occurring at physiologic and pathologic plasma iron concentrations. Normal levels of non-Tf bound iron in plasma varies between 0-1 uM (25). Patients with hereditary hemochromatosis have significantly higher levels of plasma iron, usually between 5-15 uM (55). At 1 uM ^{59}Fe, we found a 77-fold difference in uptake in the presence of PDN. Greatest uptake occurred at a 5 uM concentration of iron at which point there was a 102-fold increase in iron uptake in PDN-exposed cells. This range of response correlates well with alleged plasma levels of iron in pathologic states of overload.

It has been suggested that the mechanism of photodegraded nifedipine is independent of both transferrin and non-transferrin mediated iron uptake pathways. In

experiments with gallium, an iron analogue, TfR+ and TfR- cells demonstrated equal degrees of independent uptake of ^{67}Ga and of stimulation of uptake by PDN (76). Savigni et al found that PDN stimulated uptake of free iron, but not transferrin-Fe in erythroid cells (76). We attempted to eliminate the participation of transferrin-mediated iron uptake by preincubating cells with DMEM to deplete cellular transferrin. The question then remained of whether the enhancement of iron uptake mediated by PDN occurred due to actions on preexisting mechanisms of non-transferrin iron uptake. Our investigation of iron uptake under conditions of variable pH suggests that this is not the case. The pH optimum for control cells, representative of non-transferrin mediated uptake, was different from that in the presence of PDN. This suggests that the two pathways for iron uptake are unique. A nearly identical phenomenon has been shown in pH studies of iron uptake in erythroid cells (75). Additionally, stimulated uptake of iron in PDN exposed erythroid cells occurs in the presence of potent inhibitors of Fe (II) uptake. These findings lead to the proposition that PDN functions as a mobile iron ionophore (76). The inhibition experiments should be tested in keratinocytes to confirm a similar mechanism of action for PDN in this cell type.

We performed a series of experiments in stratified cell layers to investigate the effect of cellular differentiation on iron uptake. The iron uptake exhibited by these stratified cells is qualitatively comparable to that seen in the keratinocytes monolayers. During an incubation of 20 minutes in the presence of PDN, stratified cells augmented iron uptake by 16.4-fold, as measured by cpm/ug protein to correct for increased cell number. A similar 30 minute incubation of a proliferating monolayer demonstrated an 18-fold response (cpm/ug protein) to PDN. Essentially, then the two cell types exhibited

the same response to iron in the presence of PDN. Differentiation state does not seem to affect the *mechanism* of iron transport. In studies of fibroblasts plated at different densities, Kaplan et al demonstrated that rate of non-transferrin iron transport was not affected by cellular growth state or mitogenic activity level (44).

The *degree* to which differentiated cells participate in iron uptake, however, remains to be fully elucidated. Because of the variation in incubation time it is not possible to make conclusive statements about of the suprabasal component of the stratified cell layers. Our comparison of intact, stratified cell layers to detached suprabasal cells does allow some deduction. Uptake in stratified cells greatly exceeded that in suprabasal cells layers (16.4-fold vs. 3.7-fold). The disparity could be explained in one of two ways. On one hand, it is possible that the suprabasal cells were unhealthy and thus cell homeostasis and energy production was compromised, limiting iron uptake. Alternately, if one assumes that the suprabasal layers were healthy and functioning, then the main difference between the suprabasal cells and the stratified cells is the presence of the basal layer in the latter. These basal, proliferating cells could have been responsible for the large percentage of iron uptake, with little occurring in the overlying cell layers.

There is also the possibility that contaminating basal cells remained attached to the detached suprabasal layers. Other means of inducing cellular differentation might clarify matters by reducing these confounding factors. Methotrexate-induced differentation would eliminate the persistence of proliferating cells in the intact stratified cell layers. This would provide a source of uncontaminated differentiated cells which could then be compared to proliferating ones.

Some quantitative considerations are important in determining the feasibility of using epidermal desquamation to prevent or ameliorate iron overload (see Table 1). The numbers show that a ten-fold increase in iron elimination through the skin (0.25 mg/day to 2.5 mg/day) would adequately prevent body iron accumulation in hereditary hemochromatosis. Studies of iron overload suggest that some of that increase is already provided physiologically. Epidermal iron delivery increases 3-fold when transferrin is saturated (64). In patients with hereditary hemochromatosis, epidermal iron losses increase above normal levels, although they do not compensate for unregulated intestinal iron absorption. So the actual increase necessary through pharmacologic intervention is probably less than the predicted 2-3 mg per day. In thalassemia major it is clear that the iron burden of nearly 60 mg per day would probably be unmatched by dermato-remediation. Therefore, a reduction in the frequency of desferal infusion, rather than complete prevention, is a more reasonable therapeutic goal.

TABLE 1. QUANTITATIVE CONSIDERATIONS

Iron absorption/excretion

In normals

Absorbed iron – 1 mg/d

Excreted iron – 1 mg/d

- Feces – 0.6 mg/d
- Urine – 0.15 mg/d
- Skin – 0.25 mg/d

In hereditary hemochromatosis

Absorbed iron – 4 mg/d

* therefore, need to remove extra 2-3 mg/d

In transfusion iron overload (for thalassemia major):

Patients receive 2 units RBC/wk = 400 mg iron

Normal cellular iron content

Liver – 2.2 mg/gm (dry weight)

Epidermis – .03-0.3 mg/gm (dry weight)

Protein loss through keratinocyte desquamation:

Normal – 1 gm/day (dry weight)

Erythroderma – 12-20 gm/day (dry weight)

One study from the literature presents a challenge to our postulation that any iron we are able to load into basal keratinocytes will be lost to desquamation 26-28 days later. A study of intradermally injected radioiron, most of which was subsequently taken up by the epidermis, found that the turnover rate of iron was slower than that of the cells. Radiolabelled epidermal iron demonstrated a half-life of 67 days, whereas cellular turnover was complete in 26 days (88). In attempting to explain the mechanism of this retention the author alluded to a paper citing the extrusion and reabsorption of DNA by mature superficial epidermal cells (89). We, on the other hand, could find no such data about nucleic acid reutilization in this reference or elsewhere.

This evidence would suggest that calculations of skin iron loss based on the assumption that iron turnover equals epidermal renewal time overestimate actual skin

iron losses. However, Cavill makes a tacit assumption that all of the intradermally injected iron that was not cleared rapidly by plasma or lymph, entered the epidermis directly. He did not investigate the possibility that iron was retained in the dermis, either interstitially or bound to protein, was not investigated. The persistence of a pool of radioactive iron is supported by the Cavill's own data which show an extremely slow total body clearance of iron, lasting approximately 2 months. If some of the intradermally injected iron was trapped in the dermis, then the time to its ultimate entrance into the epidermis and subsequent desquamation would have exceeded epidermal turnover time.

Would keratinocytes be capable of safely accumulating this iron burden? The liver stores up to 2.2 mg iron per gram tissue without showing signs of significant toxicity. However, studies of patients with iron overload, suggest that different tissue types have different thresholds of iron toxicity. Iron content in the liver of a thalassemia double-transplant patient were four-fold the iron content of the heart (47). What about the sensitivity of the skin to elevated iron content? There is evidence that in states of overload, iron exerted a mild tumor promoting activity in mouse skin by enhancing oxidative stress (90). Iron's ability to generate reactive oxygen species has similarly been implicated in UV-induced skin damage (91, 87). Hyperpigmentation and dry skin are the only other cutaneous abnormalities ever reportedly linked to iron overload (14). Despite this information, the literature fails to any reports of increased skin tumorigenesis in HH. Clearly future experiments would have to investigate whether optimum conditions for increased skin iron uptake lead to the development of gross clinical signs of cutaneous

toxicity, including scaling, lichenification, atrophy, erythema, and increased incidence of skin tumors.

Our results give support to the notion that iron content of keratinocytes can be significantly modified by exposure to PDN *in vitro*. The pivotal next step in proving clinical applicability would involve investigations using animal models. It must be shown that 1) PDN administered in vivo increases iron in the epidermis; 2) PDN can be administered in a way that selectively increases iron the epidermis. Experiments will be done in hairless mice (HRS/J hr/hr). Such mice are immunologically intact and are essentially normal but for the absence of hair. Kathryn Morton has shown that intraperitoneal (i.p.) injections of PDN increases gallium uptake up to 20-fold in several internal organs (personal communication). Our initial experiments will seek to replicate this model using the i.p. route to compare the effects of dosing-regiments on iron accumulation.

The next goal would be to target the effects of PDN to the epidermis. The most logical approach to this would involve the topical application of PDN. Alternately, nifedipine could be used as a pro-drug administered systemically and activated locally to the epidermis by irradiating the skin. Using the guidance of data on parenteral dosing regimens and existing data on blood flow through the skin, we will applying PDN or its active ingredient in ethanol to one flank of hr/hr mice. Four to five different dose schedules can be applied to one flank with the opposite flank serving as an internal control. To control for the possibility of systemic absorption, a separate group of mice will serve as additional controls.

In summary we put forth the idea of a novel pharmacologic approach to the treatment of iron overload diseases. Our *in vitro* tissue culture data clearly demonstrates the potential of PDN to alter iron uptake in keratinocytes and it has helped characterize the parameters of the uptake. We are hopeful that our proposed studies *in vivo* will advance this pursuit by illustrating clinically applicable results.

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